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(54) Pharmaceutical composition which contains YC-1 as an effective component

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2□ **Abstract**

The present invention is directed to a pharmaceutical composition comprising YC-1 as an effective component.

YC-1 comprised in the pharmaceutical composition of the present invention inhibits HIF-1 activity under hypoxia. In doing so, it inhibits the expression of 3□ hypoxia-responsive genes that induce survival of cancer cells under hypoxic condition,

such as VEGF which induces angiogenesis essential for tumor proliferation and metastasis, or EPO which stimulates tumor proliferation.

Thus, the pharmaceutical composition of the present invention comprising YC-1 as an effective component can be used as an effective component for an anticancer agent.

□ The pharmaceutical composition of the present invention comprising YC-1 as an effective component can avoid resistance developing during a conventional anticancer treatment, and therefore it can be also used as an auxiliary agent for anticancer treatment such as radiation therapy, hormone therapy, chemical therapy and biological reaction regulation.

□□ Further, the pharmaceutical composition of the present invention comprising YC-1 as an effective component can be used for the treatment of disorders or conditions associated with excessive angiogenesis, the over-expression of VEGF, EPO, HIF-1 or HIF-1α which is a constitutional protein of HIF-1, and/or oxygen sensing pathway in which transition metals are involved.

□□

Representative Drawing: Fig.8

Specification

2□ **Brief descriptions for the drawings**

Figure 1 represents that YC-1 inhibits expression of VEGF and EPO genes in hypoxic condition.

Figure 2 represents YC-1 blocks HIF-1 activity in hypoxic condition.

In Figure 3, **a** and **b** represent YC-1's effect on the accumulation of HIF-1α under normoxic and hypoxic conditions: **c** and **d** represent SNP's effect on the accumulation of HIF-1α under normoxic and hypoxic conditions.

Figure 4 represents YC-1 inhibits the expression of HIF-1α in the post-transcription level in hypoxic condition.

In Figure 5, **a** represents how transfer metal ion affect the inhibitory effects of YC-1 and SNP on the expression of HIF-1α, and **b** represents how anti-oxidizing agent affect the inhibitory effect on the expression of HIF-1α in hypoxic condition by YC-1 and SNP.

In Figure 6, **a** represents how ODQ and MB affect on YC-1's inhibitory effect on HIF-1α in hypoxic condition, **b** represents how ODQ and MB affect on SNP's inhibitory effect on HIF-1α in hypoxic condition.

Figure 7 represents how cGMP affect the inhibitory effect of YC-I on the expression of HIF-1α in hypoxic condition.

Figure 8 represents that YC-1 actually has an effect of inhibiting growth and metastasis in the cancer tissue.

Detailed Description of the Invention

Purpose of the invention

Techniques relevant to the invention and prior art thereof

The present invention is directed to a pharmaceutical composition which comprises YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole] as an effective component.

Main causes of death for cancer patients are due to metastasis of cancer, in which the term 'metastasis' means that the cancer cells migrate to the places other than they originally occur, and then proliferate and propagate in the new places.

Conventional anticancer agents are usually developed to hamper various metabolic pathways of cancer cells, i.e., to inhibit tumor growth and proliferation. However, since such anticancer agents cannot block the metastasis of the cancer cells itself, the survival rate for the cancer patients using the agent is not so high.

In this connection, a need for developing a new anticancer agent, which can simultaneously inhibit tumor proliferation and metastasis, has been increasing. Recently, an angiogenesis inhibitor is getting attention as a candidate for the anticancer agent.

Angiogenesis plays two important roles in cancer. First, it provides nutrients and oxygen that are required for tumor growth and proliferation. Second, newly generated capillary blood vessels that spread into tumor tissues provide an opportunity for the cancer cells to make an entry into the blood circulation system of a patient, and therefore enabling the cancer cells to spread to the whole body of patient (i.e., metastasis).

Thus, if an agent that can inhibit the angiogenesis is used for the anticancer treatment, the growth of vascular endothelial cells surrounding the rapidly growing cancer cells can be prohibited, and therefore inhibiting tumor proliferation and metastasis. In addition, since the vascular endothelial cells surrounding normal cells usually do not proliferate, the anticancer agent comprising the angiogenesis inhibitor as an effective component hardly affects normal tissues. As such, the damage caused by the conventional anticancer agent can be avoided.

Clinical studies for anti-angiogenesis agent so far have utilized materials which can inhibit the growth and migration of endothelial cells or drug preparations which have inhibitory effect on the growth stimulation factors for the endothelial cells.

For the materials which can inhibit the growth and migration of endothelial cells, mention can be made to angiostatin and endostatin that are currently under clinical study, platelet factor-4, interleukin-12, retinoic acid, TIMP (tissue inhibitors of metalloproteinase)-1 and TIMP-2, etc.

On the other hand, for the materials which can induce the growth and migration of endothelial cells, mention can be made to VEGF (vascular endothelial growth factor), EGF (epidermal growth factor), angiogenin, estrogen, aFGF (acidic fibroblast growth factor) and bFGF (basic fibroblast growth factor), etc.

Among the above-described growth factors, particularly, VEGF is secreted in a great amount in cancer tissues, thereby maintaining the constant growth of cancer cells.

Therefore, a material which can inhibit the expression of VEGF can be a strong candidate for blocking tumor proliferation and metastasis with an inhibition of the angiogenesis.

Meanwhile, another problematic material which is secreted in a great amount by cancer cells is EPO (erythropoietin), which is a hormone stimulating the formation of erythrocytes. As the amount of EPO increases in tumor tissues, more erythrocytes are produced to provide nutrients and oxygen to the cancer cells, and therefore helping the proliferation of the cancer cells.

As such, if the expression of VEGF and EPO which are secreted from the tumor tissues and separately induce the tumor proliferation and metastasis can be inhibited, the proliferation and metastasis of cancer cells can be effectively blocked. Many

extensive studies are under way now to find out its regulation mechanism.

Expression of the genes of VEGF and EPO is known to be induced more when oxygen tension is low. Their mRNAs are induced much higher under low-oxygen condition (i.e., hypoxia). Chronic hypoxia is a very typical condition found in the tumor tissues. Expression of certain genes including said VEGF and EPO under hypoxia is mediated by HIF-1 (hypoxia-inducible factor). HIF-1 consists of two proteins, HIF-1α and HIF-1β. At the protein level, HIF-1β protein which is constitutively present regardless of oxygen tension, whereas, HIF-1α protein is markedly increased by hypoxia. Under normoxia, HIF-1α is rapidly degraded. Therefore, the stability of HIF-1α according to oxygen tension regulates the HIF-1 activity.

The stability of HIF-1α is also affected by some factors that are involved with the oxygen sensing pathway besides oxygen tension. Such factors include transition metal ions, iron chelators and antioxidants, etc.

Furthermore, the stability of HIF-1α is affected by NO (nitric oxide). If hypoxia continues, NO is produced by iNOS (inducible nitric oxide synthetase). NO inhibits the accumulation of HIF-1α. As a result, HIF-1 cannot bind to regulation site of a target gene, thereby inhibiting the expression of VEGF and EPO under the hypoxia. Consequently, the tumor proliferation and metastasis are blocked by NO.

Another effect of NO is to increase the concentration of cGMP (cyclic guanosine monophosphate) by activating sGC (soluble guanylate cyclase) with its binding to the iron molecule of the heme structure of sGC. That can cause vasodilation and cytotoxicity. There is one hypothesis that the inhibitory effect of NO on HIF activity under hypoxia is also mediated by the increase of cGMP through the sGC activation. Other hypotheses were presented in which NO may bind to a molecule that can regulate

the stability of HIF-1α or may regulate the factors involved with the oxygen sensing pathway. More studies are in need to confirm such hypotheses.

Even if NO can significantly inhibit the expression of the factors involved with tumor proliferation and metastasis, it may cause damage to the tissue due to its very high reactivity. As such, it is unsuitable to be used for a clinical treatment.

Therefore, there is strong need to develop a material which inhibits the expression of VEGF and EPO, that play an important role in proliferation and metastasis of tumor tissues, but has no adverse effect on normal tissues.

Technical subject of the invention

An object of the present invention is to provide a pharmaceutical composition which can effectively inhibit both tumor proliferation and metastasis without any adverse effect.

Technical constitution of the invention

The present invention provides a pharmaceutical composition which has an anticancer activity by inhibiting the expression of VEGF and EPO under hypoxia.

YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole] comprised in the pharmaceutical composition of the present invention inhibits the HIF-1 activity under hypoxia, and therefore inhibiting the expression of EPO and VEGF. YC-1 comprised in the pharmaceutical composition of the present invention also inhibits the accumulation of HIF-1α at post-translational level, thus inhibiting the synthesis of HIF-1

protein. Consequently, the activity of HIF-1 protein under hypoxia is inhibited.

It is believed that YC-1 comprised in the pharmaceutical composition of the present invention regulates the HIF-1 activity by blocking the oxygen sensing pathway mediated by the transition metals, and this is different from NO which has the same □ physiological effect as HIF-1.

Thus, since YC-1 of the present invention has a strong anticancer effect by inhibiting the expression of EPO and VEGF that assist tumor proliferation and metastasis under chronic hypoxia without any adverse effects of highly-reactive NO, YC-1 can be used as an effective component for an anticancer agent.

□□ The pharmaceutical composition of the present invention comprising YC-1 as an effective component can avoid any resistance developing during the conventional anticancer treatment, and therefore can be used as an auxiliary agent for an anticancer treatment such as radiation therapy, hormone therapy, chemical therapy and biological reaction regulation.

□□ Further, the pharmaceutical composition of the present invention comprising YC-1 as an effective component can be used for the treatment of disorders or conditions associated withexcessive angiogenesis, the overexpression of VEGF, EPO, HIF-1 or HIF-1α which is a constitutional protein of HIF-1, and/or transition metal-mediated oxygen sensing pathway.

2□ The pharmaceutical composition of the present invention comprising YC-1 as an effective component can be used for the treatment of diseases related to excessive angiogenesis and overexpression of VEGF and HIF-1 such as cardiovascular diseases including angioma, angiofibroma, vascular malforamtion, arteriosclerosis, vascular adhesion and scleroedema; ocular disease including angiogenesis for corneal transplant,

angiogenesis-related glaucoma, diabetic retinosis, corneal diseases, macular degenration, pterygium, retinal degeneration, retrolental fibroplasia and granular conjunctivitis; chronic inflammatory diseases including arthritis; and skin diseases including psoriasis, telangiestasia, pyogenic granuloma, seborrheic dermatitis and acne.

In addition, the pharmaceutical composition of the present invention comprising YC-1 as an effective component can be used for the treatment of disease related to the overexpression of EPO and HIF-1, such as erythrocytosis and kidney failure, etc.

Furthermore, the pharmaceutical composition of the present invention comprising YC-1 as an effective component can be used for the treatment of diseases that are related to transition metal ions-mediated oxygen sensing pathway, such as heart attack, premature aging and diabetes, etc.

The pharmaceutical composition of the present invention can be prepared by combining YC-1 with a pharmaceutically acceptable carrier. The pharmaceutical composition of the present invention can further comprise general additives such as excipient, disintegrant, sweetener, lubricant or flavoring agent. In accordance with a conventional method, the present composition can be formulated into tablets, capsules, powders, granules, suspensions, emulsions, syrups or other liquid formulations. The pharmaceutical composition of the present invention comprising YC-1 as an effective component is relatively non-toxic.

The pharmaceutical composition of the present invention can be administered to a patient in need of treatment via oral-, rectal-, topical-, intravenous-, intraperitoneal-, intramuscular-, intraarterial-, transdermal-, intranasal-, intraocular- or intradermal route, or by inhalation.

The dosage of the composition according to the present invention may be within

a range of 0.3 – 3000mg/kg of body weight. The frequency of administration is preferably 1 to 3 times per day. The dosage and frequency of administration may vary depending on body weight of the recipient, age, sex, overall physical condition, diet, administration time and method, excretion rate of the recipient and severity of illness, □ etc.

The invention is further exemplified by means of the following examples. However, the present invention in any case is not limited to those examples.

□□ **[Example 1]**

Inhibitory effect of YC-1 on the expression of VEGF and EPO genes under hypoxia

Experiments were carried out in order to examine whether or not YC-1 inhibits the expression of VEGF and EPO genes under hypoxia.

□□ Hep3B cells, a hepatoma cell line, were treated with YC-1 in the concentration of 100 or 200 μM. For the positive control, NO-releasing SNP (sodium nitroprusside) was added to Hep3B cells in the concentration of 5 or 10 μM. Treated cells were cultured for 16 hr under the normoxic- (140 ㎜Hg, 20% O_2, v/v) and the hypoxic condition (7 ㎜Hg, 1% O_2, v/v), respectively.

2□ In order to isolate total RNA from the cells prepared in the above, TRIZOL kit commercially available from GIBCO/BRL was used. The mRNA expressions were measured using Semi-quantitative RT-PCR (reverse transcriptase - polymerized chain reaction).

□□

RT reaction solution comprises 1㎍ total RNA, 1×AMV (avian myeloblastosis virus) reaction buffer, 0.2 mM dNTPs and 0.1 unit/㎖ AMV reverse transcriptase in 50㎕ volume. The reaction was carried out for 1 hr at 48 ℃.

After obtaining cDNA with the RT reaction described in the above, PCR was carried out in the presence of 1 × Tfl reaction buffer, 5 μCi[α-^{32}P]CPT, 1.5 mM magnesium sulfate, 0.1 unit/㎕ Tfl DNA polymerase enzyme and 250 nM primers. The primers used in this reaction are prepared so as to amplify the genes of EPO, VEGF and β-actin, respectively, and their sequences are described in the following table 1.

[Table 1]

Subject gene to be amplified	Forward primer	Reverse primer
EPO	CTGGAGAGGTACCTCTTGGA (SEQ ID No. 1)	CCTGTGTACAGCTTCAGCTT (SEQ ID No. 2)
VEGF	AACTTTCTGCTGTCTTGG (SEQ ID No. 3)	TTTGGTCTGCATTCACAT (SEQ ID No. 4)
β-actin	AAGAGAGGCATCCTCACCCT (SEQ ID No. 5)	ATCTCTTGCTCGAAGTCCAG (SEQ ID No. 6)

PCR condition was as follows; 20 cycles of denaturation at 94℃ for 30 sec, annealing at 56℃ for 30 sec, and enlogation at 70℃ for 1 min. The resulting PCR fragments were electrophoresed on 4% polyacrylamide gel at 100V in a 0.3 ×TBE

buffer solution (15 mM Tris and 30 mM boric acid), 0.06 mM EDTA, pH7.5) at 4℃. After completing the electrophoresis, the gel was dried. Subsequently, the mRNAs were quantified and compared to each other using auto-radiography. The results are shown in **Fig. 1**.

□ As shown in **Fig. 1**, β-actin, which has a constant expression rate in living cells, had quite steady expression of its mRNA, regardless any variables such as oxygen tension, SNP- or YC-1 concentration.

On the other hand, EPO and VEGF genes were hardly expressed under the normoxic condition, while their expression was significantly induced under the hypoxic

□□ condition so that their mRNA levels were significantly increased. These are in consistent with previous known results.

When the cells were treated with YC-1 under the hypoxic condition, the expression of their respective mRNAs was suppressed as same as the cells treated with NO. Such inhibitory effect was more prominent at higher concentration of YC-1.

□□ This result indicates that the hypoxic induction of EPO and VEGF mRNAs is suppressed by YC-1 similar to NO in the cells.

[Example 2]

<u>Inhibitory effect of YC-1 on the HIF-1 activity under hypoxia</u>

2□ **1) Inhibitory effect of YC-1 on the HIF-1 activity under hypoxia**

In accordance with the following experimental results, it was confirmed that YC-1 suppressed the expression of EPO and VEGF genes under hypoxia by inhibiting the HIF-1 activity.

□ EMSA (electrophoretic mobility gel shift assay) was carried out to examine the effect of YC-1 on the binding affinity between EPO gene and HIF-1 under hypoxia. In order to obtain nuclear extracts, Hep3B cells were cultured similarly as in the above-described RT-PCR experiment. The Hep3B cells were cooled by placing the plates on ice and were then washed twice in ice-cold PBS. After scraping the cells from the □□ plates, they were centrifuged at 1000 *g* for 5 min at 4℃, and then twice washed with PBS. The pellet was resuspended in a lysis buffer containing 0.2 % Nonidet P-40, and the nuclear extract was obtained (Chun et al., Biochem. Biophys. Res. Commun., 268:652-656, 2000). The probe used in EMSA analysis was labeled using [γ-^{32}P]ATP and its nucleotide sequence is as follows:

□□ 5' - ACCGGCCCTACGTGCTGTCTCAC - 3' (SEQ ID No. 7)

DNA-protein binding reactions were carried out for 20 min at 4℃ in a total volume of 20 ㎕, containing 5 ㎍ of nuclear extract, 0.4 ㎍ of sonicated, denatured calf thymus DNA and 1×10^4 cpm of oligonucleotide probe in EMSA reaction mixture 2□ consisting of 10 mM Tris (pH 7.4), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 5 mM DTT (dithiothreitol) and 5 % glycerol. For supershift analysis, 1 ㎕ of rat HIF-1α antiserum was added to the completed EMSA reaction mixture and incubated for 2 hr at

4 ℃.

After the reaction completed, the resultant was subjected to the electrophoresis on 5 % non-denaturing polyacrylamide gel. Electrophoresis was performed at 200V in 0.3 ×TBE buffer solution at 4℃. Results were confirmed based on auto-radiography (**Fig. 2**).

As known previously, no HIF-1/DNA complex was observed under normoxia. However, under hypoxia, the band corresponding to the HIF-1/DNA complex (HIF-1 band) was detected above non-specific complex band (C). Such band originates from the complex formed by binding HIF-1 α antiserum to HIF-1/DNA complex, thus it has higher molecular weight than the HIF-1/DNA complex itself. This result again confirms the binding between HIF-1 and DNA under hypoxia.

Meanwhile, when the cells were treated with 100 μM of YC-1 under hypoxic condition, HIF-1/DNA binding was inhibited, similar to the positive control group treated with 5 μM of SNP. The extent of such inhibition is similar to that under normoxia.

Thus, similar to NO, YC-1 inhibits the binding of HIF-1 to the regulation site of a target gene, thereby suppressing the expression of EPO and VEGF.

2) Inhibitory effect of YC-1 on the HIF-1α accumulation under hypoxia

In accordance with the following experimental results, it was confirmed that YC-1 inhibited the accumulation of HIF-1α, a constitutional protein of HIF-1, under hypoxia, thereby inhibiting the binding between HIF-1 and DNA.

Hep3B cells were treated with YC-1 or SNP in various concentrations of 0, 0.002, 0.01, 0.02, 0.05, 0.25, 1, 2.5, 5, 25, 100, or 200 μM. The treated cells were exposed to hypoxia or normoxia. Then cell extracts were obtained.

For Western blot analysis, 20 μg of cell extract was separated on 4 % SDS/polyacrylamide gel, and transferred to an Immobilon-P membrane manufacture (Milipore).

The membrane was incubated overnight at 4℃ with rat anti-HIF-1 α, diluted 1:5000 in TBS/0.1% Tween 20(TTBS) containing 5% nonfat milk. After extensive washing with TTBS, the membrane was incubated for 1 hr with horseradish peroxidase-conjugated sheep anti-rat antiserum, diluted 1:5000 in TTBS containing 5% nonfat milk. After washing, the complexes were visualized using ECL+(enhanced chemiluminescence plus, Amersham).

Fig. 3a demonstrates that the protein level of HIF-1α is hardly present under normoxia, while greatly increases under hypoxia, as known previously. When the cells were treated with YC-1 at 25 μM or less than under hypoxic condition, HIF-1α level was unaffected. However, 100 μM or more than of YC-1 inhibited the hypoxic accumulation of HIF-1α. In contrast, the density of a non-specific protein band (NS) was unaltered by YC-1 treatment, which suggests that YC-1 specifically inhibits the accumulation of HIF-1α. Under the normoxic condition, YC-1 had no effect on the accumulation of HIF-1α (**Fig. 3b**).

For the positive control treated with SNP, the inhibitory effect of SNP on the hypoxic accumulation of HIF-1α was observed in accordance with the increase of its

concentration (**Fig. 3c**). Under the normoxic condition, there was no effect on the accumulation of HIF-1α (**Fig. 3d**).

3) Inhibitory effect of YC-1 on the HIF-1α accumulation under hypoxia at post-translational level

In accordance with the following experimental results, it was confirmed that the inhibitory effect of YC-1 on the HIF-1α accumulation under hypoxia occurred at post-translational level.

In order to examine the effect of YC-1 and SNP on the expression of HIF-1α mRNA under hypoxia, semi-quantitative RT-PCR was carried out according to the same method described in Example 1. The primers used for this experiment were designed to amplify HIF-1α. Their nucleotide sequences are described below:

5' - CCCCAGATTCAGGATCAGACA - 3' (SEQ ID No. 8)

5' - CCATCATGTTCCATTTTTCGC - 3' (SEQ ID No. 9)

As shown in **Fig. 4**, the HIF-1α mRNA levels under hypoxia were unaffected by treatment with YC-1 or SNP, regardless of their individual concentration.

Such results indicate that YC-1 inhibits the HIF-1 α activity by blocking the hypoxic accumulation of HIF-1 α at post-translational level, not at transcription level.

[Example 3]

Inhibitory effect of YC-1 on the oxygen sensing pathway related to the transition metals

Hypoxic accumulation of HIF-1 α is affected by factors which are related to the oxygen sensing pathway, such as transition metals and intracellular redox state. In this connection, experiments were carried out to examine whether the YC-1 affects these processes that regulate the HIF-1α accumulation under hypoxia.

In order to examine any effect by the transition metals and intracellular redox state, Hep3B cells were treated with cobalt ion, iron chelator (desferroxamine) and antioxidants (trolox and N-acetyl cysteine), respectively. Hep3B cells were treated with 75 μM cobalt chloride, 120 μM desferroxamine, 5 μM trolox and 20 mM N-acetyl cysteine (NAC), respectively. Then, the resulting cells were treated further with either 100 μM YC-1 or 5 μM SNP. After obtaining the cell extracts, Western blot analysis was carried out according to the same method described in Example 2. The results are given in **Fig. 5**.

As shown in **Fig. 5**, both cobalt and desferroxamine induced the HIF-1α accumulation of under normoxic condition (lane 2 and 6). However, such effect was inhibited by YC-1 (lane 3 and 7). SNP had no effect on the transition metals-induced accumulation of HIF-1 α (lane 4 and 8).

On the other hand, the antioxidants, trolox and NAC, failed to recover HIF-1 α levels (lane 3 and 6) suppressed by YC-1 and SNP, as shown in **Fig. 5** (lane 4, 5, 7 and 8).

Therefore, it is believed that suppression mechanism of YC-1 on the HIF-1α

accumulation under hypoxia is linked with the metal ion-related pathway of oxygen sensing, but not with the cellular redox state. In addition, considering that NO having a similar physiological function seems to work independently from the transition metals, YC-1 may regulate the HIF-1 activity under hypoxia via a reaction mechanism that is different from that of NO.

[Example 4]

Relationship between the effect of YC-1 under hypoxia and sGC and cGMP

YC-1 has been known to increase the intracellular cGMP levels by stimulating sGC. In this connection, experiments were carried out to test whether the effect of YC-1 under hypoxia is related to this mechanism or not.

1) Relationship between the effect of YC-1 and sGC activation under hypoxia

To examine the effect of sGC on YC-1 action, ODQ [1H-(1,2,4)oxadiazole(4, 3a)quinoxatin-1-one] and MB (methylene blue), which are inhibitors of sGC, were added to HepP3B cells in an amount of 10, 20, or 50 μM. Next, the resulting cells were treated with 100 μM YC-1 or 5 μM SNP. The cells were incubated for 4 hr under the normoxic- or hypoxic conditions. Subsequently, cell extracts were prepared. Western blot analysis was carried out according to the same method described in Example 2. Results are given in **Fig. 6**.

As a result, none of ODQ or MB had any effect on the action of either YC-1 (**Fig. 6a**) or SNP (**Fig. 6b**) under hypoxia.

These results indicate that the inhibitory effect of YC-1 on the accumulation of

HIF-1α under hypoxia is based on a mechanism that is not related to sGC.

2) Relationship between the effect of YC-1 and cGMP concentration under hypoxia

To examine the effect of cGMP on YC-1 action, Hep3B cells were treated with a combined preparation of various agents that can increase or decrease cGMP concentration, in a different amount, respectively. The cells were incubated with said combination for 4 hr under the hypoxic condition. For this experiment, YC-1 and SNP were added to the media 5 min prior to 4 hr incubation of hypoxia, while NAME (Ngamma-Nitro-L-arginine methyl ester) and 8-bromo-cGMP, which are NO synthetase inhibitor and cGMP analogue, respectively, were added to the cells 1 hr before the hypoxic incubation. After obtaining the cell extracts, western blot analysis was carried out according to the same method described in Example 2. Results are given in **Fig. 7**.

As can be seen from Fig. 7, 100 μM of YC-1 significantly suppressed the accumulation of HIF-1α (lane 3) compared to the hypoxic condition (lane 2), and these are in accordance with previously results. It is confirmed that such inhibitory action of YC-1 is based on its own function, not dependent on NO concentration (lane 4). Meanwhile, when the cells were treated with less-than-functional concentration of YC-1 or SNP, no inhibition on HIF-1α accumulation under hypoxic condition was observed (lane 5 and 6). Even when the mixture of YC-1 and SNP were added to the cells to greatly increase cGMP concentration, no inhibition was observed (lane 7). Addition of cGMP analogue had also no effect on the accumulation of HIF-1α (lane 10 and 11).

Based on theses results, it is found that the inhibitory effect of YC-1 on the HIF-1α accumulation under hypoxia is not related to the sGC activation or the increased

cGMP concentration.

[Example 5]

Inhibitory effect of YC-1 on tumor proliferation and metastasis *in vivo*

□ In accordance with the following experimental results, it was confirmed that YC-1 had an inhibitory effect on tumor proliferation and metastasis *in vivo*.

Nude mice were injected subcuteneously with Hep3B cells to develop tumor. Then, YC-1 (30mg/body weight (kg)) was administered to mice once a day.

□□ It was necessary to determine whether the inhibitory action of YC-1 on tumor metastasis is effective on the early stage of tumor or on the later stage in which the tumor is established. Thus, in order to examine the effect of YC-1 on the time-lapse of tumor development, one test group was prepared in which YC-1 was administered to mice for two weeks starting from the day of the injection of Hep3B cells

□□ (1-14 days). The other test group was also prepared in which YC-1 was administered to mice for the same period of two weeks, but it began 40 days after the injection of Hep3B cells (40-53 days). Control group received no YC-1 at all. Tumor size in said test and control groups was then measured for 60 days. The results are summarized in **Fig. 8**.

2□ As can be seen from **Fig. 8**, for the test group administered with YC-1 for two weeks starting from the day of the injection of Hep3B cells, tumor size started to decrease 30 days after the injection of Hep3B cells, compared to the control group. Further, around 60 days, the tumor size for the test group was 2.5 times less than that of

the control group. For the test group administered with YC-1 for two weeks, starting from 40 days after the injection of Hep3B cells, tumor size started to decrease right after the administration, and it became 7 times smaller than that of the control group around 60 days after the injection of Hep3B cells.

□ These results indicate that YC-1 of the present invention can inhibit tumor proliferation and metastasis not only for the early stage in which tumor is developing but also for the later stage in which tumor is established.

Effect of the invention

□□ YC-1 comprised in the pharmaceutical composition of the present invention can inhibit the binding of HIF-1 to the regulation site of a target gene under hypoxia, thereby inhibiting the expression of EPO and VEGF that are activated by HIF-1. Consequently, tumor metastasis can be inhibited.

Action mechanism of YC-1 under hypoxia is related to the oxygen sensing
□□ pathway that involves the transition metals. Since the inhibitory action of YC-1 on HIF-1 is based on a different mechanism from NO, YC-1 can be used as an anticancer agent that is free of any adverse effects caused by NO.

The pharmaceutical composition of the present invention comprising YC-1 as an effective component can block the tumor metastasis not only for early stage in which
2□ tumor is developing but also for later stage in which tumor tissue is established. As such, contrary to conventional anticancer agent of which use is limited to a certain stage of tumor development, the pharmaceutical composition of the present invention can be broadly administered to a patient in any stage of tumor development.

The pharmaceutical composition of the present invention comprising YC-1 as an effective component can avoid any resistance developing during the conventional anticancer treatment, and therefore it can be used as an auxiliary agent for anticancer treatment such as radiation therapy, hormone therapy, chemical therapy and biological reaction regulation.

Further, the pharmaceutical composition of the present invention comprising YC-1 as an effective component can be used for the treatment of disorders or conditions associated withexcessive angiogenesis, the overexpression of VEGF, EPO, HIF-1 or HIF-1α which is a constitutional protein of HIF-1, and/or oxygen sensing pathway in which transition metals are involved.

What is claimed is:

Claim 1

Anti-cancer drug which contains YC-1(3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole) as an effective component.

□

Claim 2

With respect to claim 1, anti-cancer drug inhibiting cancer growth and metastasis at the same time.

□□ **Claim 3**

Angiogenesis inhibitor which contains YC-1 as an effective component.

Claim 4

VEGF(Vascular endothelial growth factor) expression inhibitor which contains YC-1 as
□□ an effective component.

Claim 5

EPO(Erythropoietin) expression inhibitor which contains YC-1 as an effective component.

2□

Claim 6

Inhibitor of HIF-1 activaty which contains YC-1 as an effective component.

Claim 7

2□ Inhibitor of HIF-1α accumulation which contains YC-1 as an effective component.

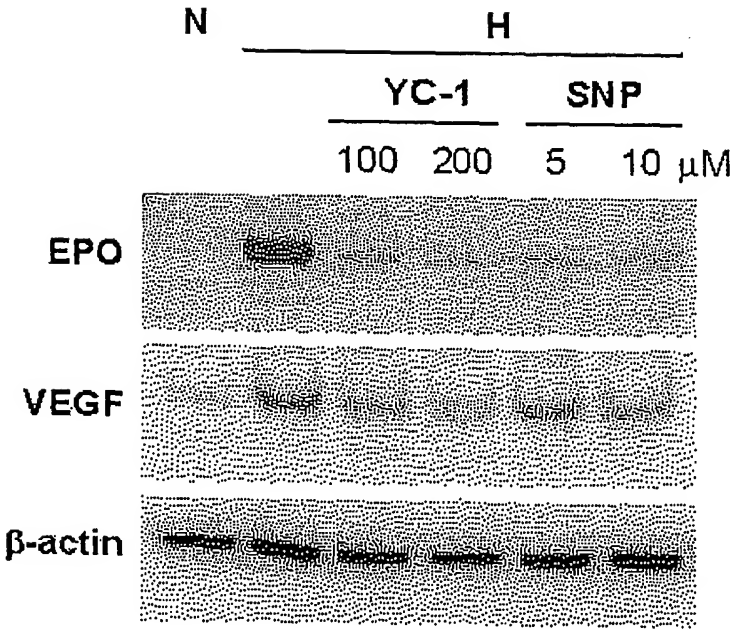
Claim 8

Inhibitor of oxygen sensing pathway related with transition metal ion which contains YC-1 as an effective component.

3□

Drawings

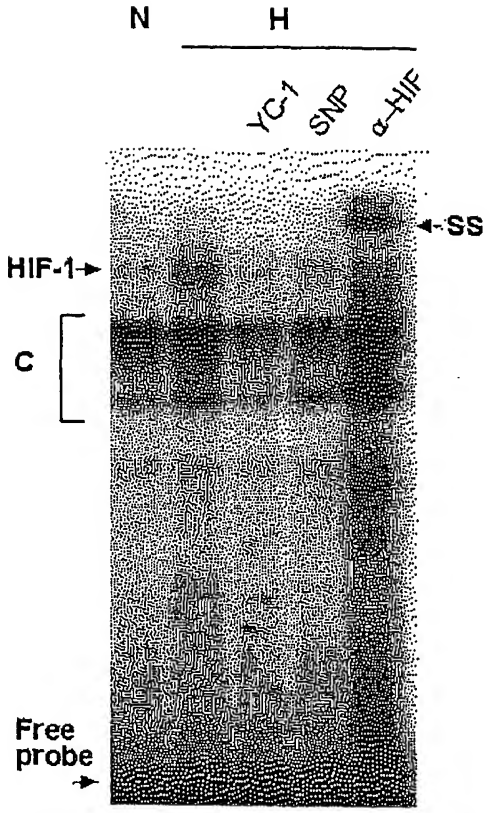
FIG. 1



N : normoxia

H : hypoxia

FIG. 2

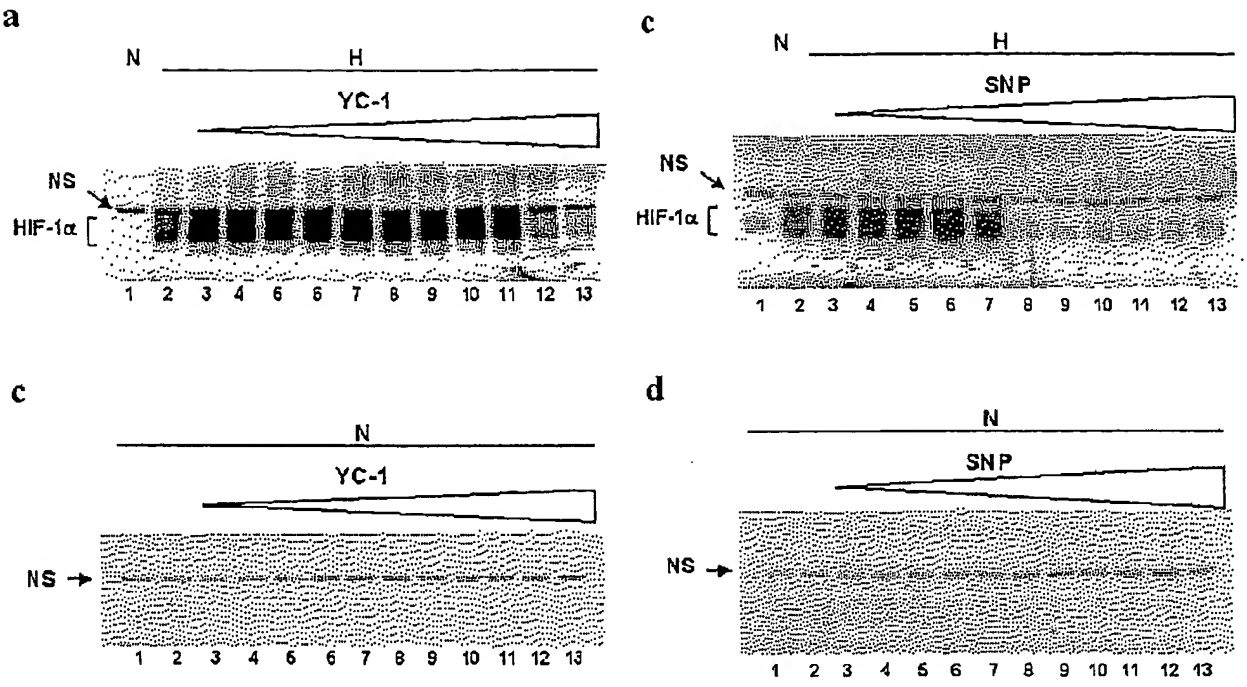


HIF-1 : HIF-1 binding

C : constitutive binding

SS : supershift band

FIG. 3



NS : nonspecific protein band

FIG. 4

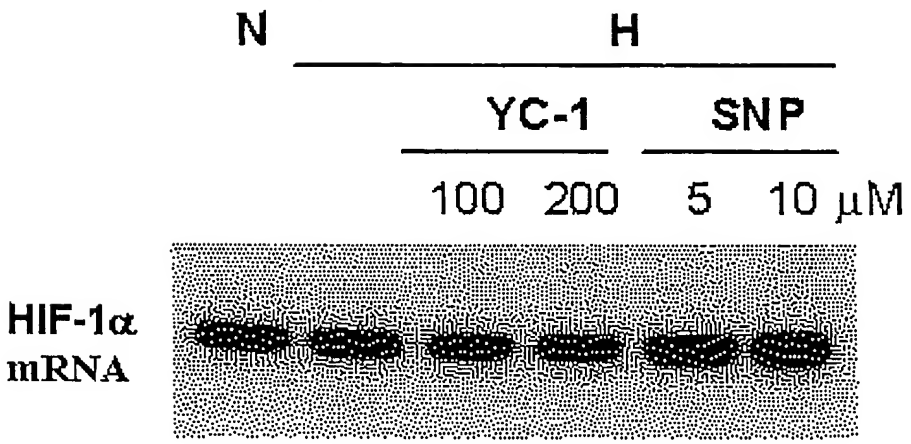


FIG. 5

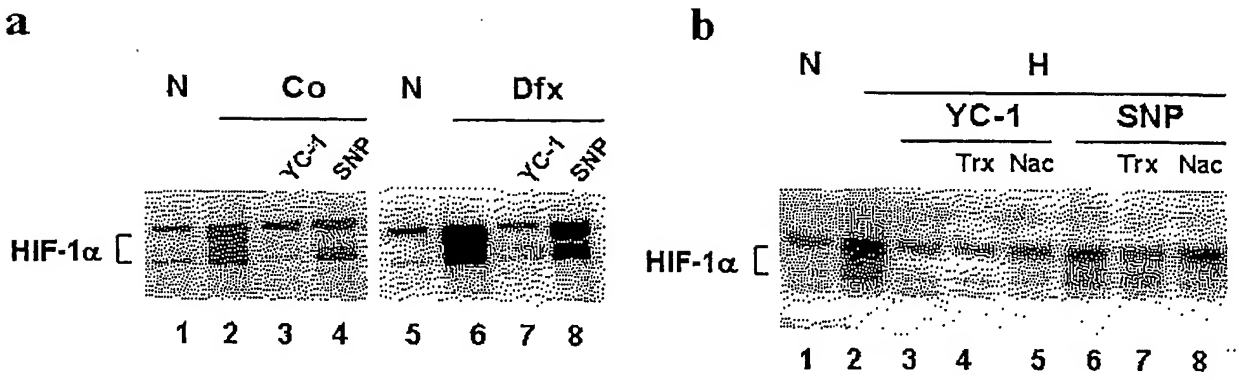
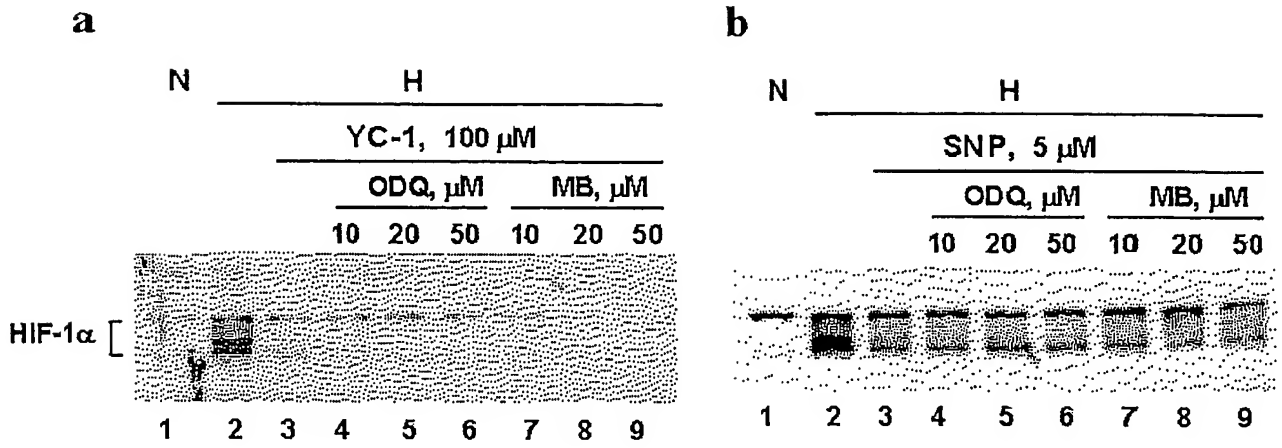

a
N
Co
N
Dfx
YC-1
SNP
YC-1
SNP
HIF-1α
1 2 3 4 5 6 7 8
b
N
H
YC-1
SNP
Trx Nac
Trx Nac
HIF-1α
1 2 3 4 5 6 7 8

FIG. 6


a
N
H
YC-1, 100 μM
ODQ, μM
MB, μM
10 20 50 10 20 50
HIF-1α
1 2 3 4 5 6 7 8 9
b
N
H
SNP, 5 μM
ODQ, μM
MB, μM
10 20 50 10 20 50
1 2 3 4 5 6 7 8 9

□

FIG. 7

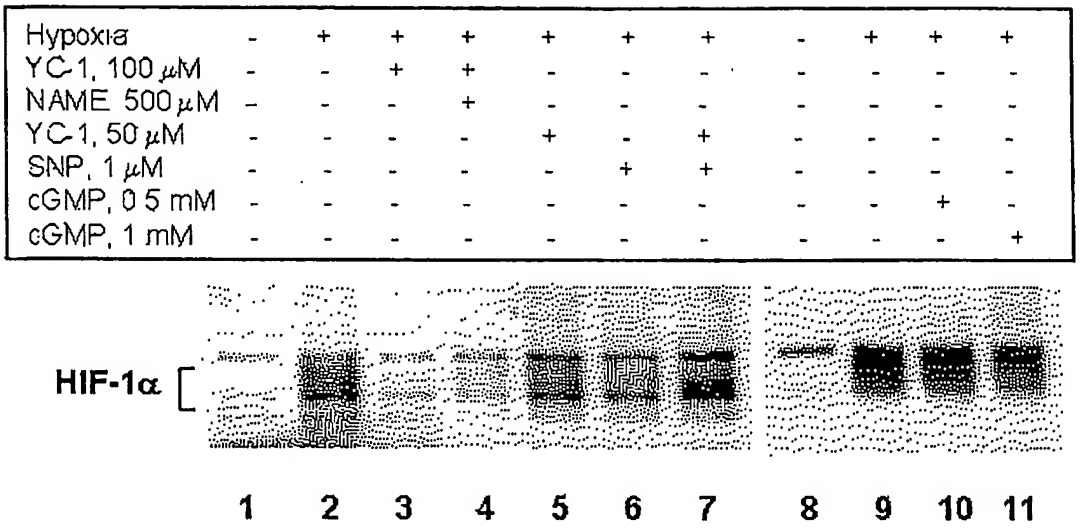
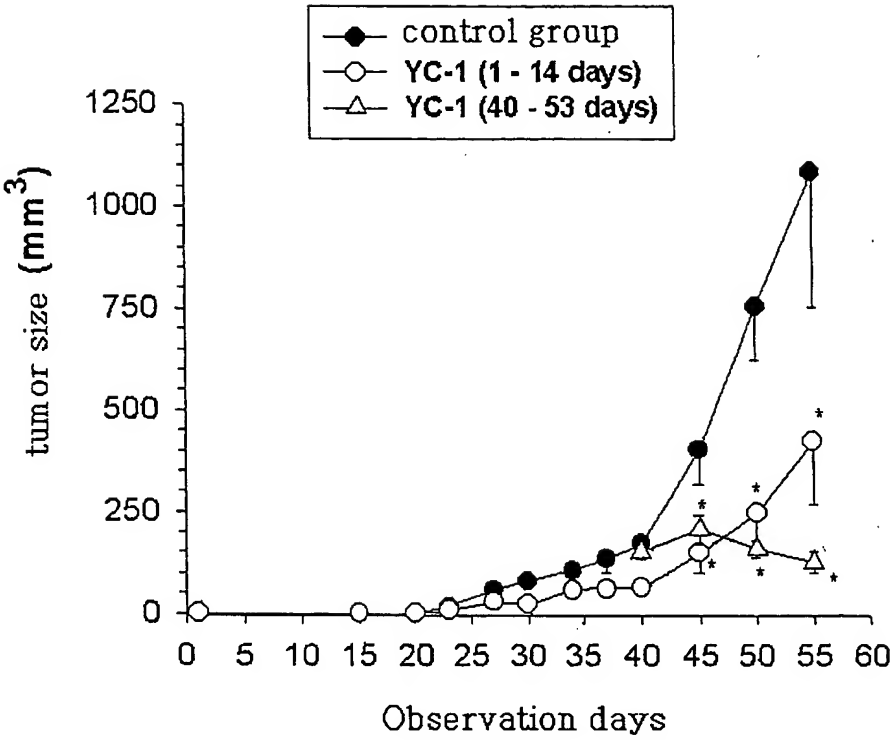

Hypoxia - + + + + + + - + + +
YC-1, 100 μM - - + + - - - - - - -
NAME 500 μM - - - + - - - - - - -
YC-1, 50 μM - - - - + - + - - - -
SNP, 1 μM - - - - - + + - - - -
cGMP, 0 5 mM - - - - - - - - - + -
cGMP, 1 mM - - - - - - - - - - +
HIF-1α
1 2 3 4 5 6 7 8 9 10 11

FIG. 8


control group
YC-1 (1 - 14 days)
YC-1 (40 - 53 days)
tumor size (mm3)
1250
1000
750
500
250
0
0 5 10 15 20 25 30 35 40 45 50 55 60
Observation days

Sequencing List

<110> PARK, Jong Wan CHUN, Yang Sook<120> pharmaceutical composition containing YC-1 as effective component<130> 01P-78<160> □ 9<170> KopatentIn 1.71<210> 1<211> 20<212> DNA<213> Artificial Sequence<220><223> forward primer for amplifying erythropoietin gene<400> 1ctggagaggt acctcttgga 20<210> 2<211> 20<212> DNA<213> Artificial Sequence<220><223> reverse primer for amplifying erythropoietin gene<400> □□ 2cctgtgtaca gcttcagctt 20<210> 3<211> 18<212> DNA<213> Artificial Sequence<220><223> forward primer for amplifying vascular endothelial growth factor gene<400> 3aactttctgc tgtcttgg 18<210> 4<211> 18<212> DNA<213> Artificial □□ Sequence<220><223> reverse primer for amplifying vascular endothelial growth factor gene<400> 4tttggtctgc attcacat 18<210> 5<211> 20<212> DNA<213> Artificial Sequence<220><223> forward primer for amplifying beta-actin gene<400> 5aagagaggca tcctcaccct 2□ 20<210> 6<211> 20<212> DNA<213> Artificial Sequence<220><223> reverse for amplifying beta-actin gene<400> 6atctcttgct cgaagtccag 20<210> 7<211> 23<212> DNA<213> Artificial Sequence<220><223> probe for EMSA assay<400> 7accggcccta cgtgctgtct cac

23<210> 8<211> 21<212> DNA<213> Artificial Sequence<220><223> forward primer for amplifying HIF-1 alpha gene<400> 8ccccagattc aggatcagac a

21<210> 9<211> 21<212> DNA<213> Artificial Sequence<220><223> reverse primer for amplifying HIF-1 alpha gene<400> 9ccatcatgtt ccatttttcg c